Supplementary Information for Manuscript

SHARPIN forms a linear ubiquitin ligase complex regulating NF-κB activity and apoptosis

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Supplementary figure legends

Figure S1. SHARPIN is a UBL and ubiquitin binding domain containing protein.

a, Schematic representation of the SHARPIN protein truncations used; SHARPIN Full-length (A), SHARPIN ΔNZF (B), SHARPIN T358L, F359V (TF_LV) (C), SHARPIN ΔUBL (D), SHARPINI272A (E). **b,** Ubiquitin pull-down assays using GST-tagged ubiquitin species of varying length to evaluate the interaction with purified His-tagged SHARPIN. GST pull downs were separated by SDS-PAGE, followed by detection by Western blot analysis. Ponceau S staining indicates the input of GST fusion proteins. **c,** GST pull-down analysis of linear tetra-ubiquitin and K63-linked tetra-ubiquitin chains, employing a panel of GST-tagged SHARPIN protein variants. GST-4xUb was purified from *E.coli*; K63-linked tetra-ubiquitin was synthesized *in vitro*. GST pull downs were separated by SDS-PAGE, followed by detection of interaction events by Western blot analysis. Ponceau S staining depicts the input of GST fusion proteins. **d**, HEK293T cells were transfected with the indicated GFP-tagged SHARPIN variants for 24 hours prior to lysis. Cell lysates were subjected to pull-down experiments with GST alone (negative control) or GST conjugated ubiquitin coupled to Glutathione Sepharose beads. **e**, HEK293T cells were transfected with the indicated HA-HOIL constructs for 24 hours prior to lysis. Cell lysates were subjected to pull-down experiments with GST alone or GST conjugated ubiquitin coupled to Glutathione Sepharose beads. **f**, GST conjugated ubiquitin was purified from *E. coli*. After removal of GST by thrombin cleavage, the purified tetra-ubiquitin was used in pull-down experiments with GST alone or GST conjugated HOIL-1L-NZF or HOIL-1L-UBL. The GST pull-downs were separated by SDS-PAGE and analyzed by Western blot using anti-ubiquitin antibodies. **g**, HEK293T cells were transfected with Myc-tagged full-length HOIP for 24 hours prior to lysis. Cell lysates were subsequently subjected to pull-down experiments with GST alone or the indicated GST conjugated ubiquitin species, coupled to Glutathione Sepharose beads. GST pull-downs were analyzed as described above. **d-g,** WCL = whole cell lysate. Ponceau S

staining depicts the input of GST fusion proteins. **h**, GST conjugated ubiquitin was purified from *E. Coli*. After removal of GST by thrombin cleavage, the purified tetra-ubiquitin was used in pull-down experiments with GST alone or GST conjugated HOIP protein variants coupled to Glutathione Sepharose beads. GST pull-downs were analyzed as described above. **i, j,** The SHARPIN UBL domain binds to the NZF2 domain of HOIP. GST pull-down analysis of His-tagged SHARPIN variants, using GST-tagged isolated domains of HOIP (e.g. NZF1, NZF2 or UBA). GST pull downs were separated by SDS-PAGE, followed by detection of interaction events by Western blot analysis. Ponceau S staining depicts the input of GST fusion proteins.

Figure S2. SHARPIN forms a trimeric complex with HOIP and HOIL-1L.

a, The interaction between SHARPIN and HOIP is dependent on an intact SHARPIN UBL domain. HEK293T cells were transfected with full-length Myc-tagged HOIP, together with either wild type or mutated SHARPIN variants, deficient in ubiquitin-binding (T358L, F359V; TF_LV) or HOIP-binding (ΔUBL or I272A), respectively. Cell lysates were subjected to immunoprecipitation using anti-Myc antibodies, followed by SDS-PAGE and Western blot analysis. 2% of total lysate input is used to indicate transfection efficiency. **b**, SHARPIN does not interact directly with HOIL-1L. GST-tagged full-length SHARPIN or tetra-ubiquitin was used to pull down purified His-tagged HOIL-1L. GST pull downs were separated by SDS-PAGE, and analyzed by Western blot. **c**, Model indicating the arrangement and ubiquitin binding domains of the three putative LUBAC complexes; a trimeric HOIP-HOIL-1L-SHARPIN (A), dimeric HOIP-HOIL-1L (B) and HOIP-SHARPIN (C). In our model SHARPIN binds to linear ubiquitin chains via its NZF domain and utilizes the UBL for a direct interaction with the HOIP NZF2. Both HOIP (NZF1) and HOIL-1L employ their NZF domains to associate with ubiquitin and interact with each other via a direct binding between

the HOIP UBA and HOIL-1L UBL domains. The UBL and NZF regions of SHARPIN and HOIL-1L are highly homologous.

Figure S3. SHARPIN-HOIP induce linear ubiquitin formation.

a, HOIL-1L and HOIP stimulate linear ubiquitylation *in vitro*. *In vitro* ubiquitylation assay using purified ubiquitin, E1, E2 (UbcH7) and E3 proteins (HOIL-1L and HOIP), in the absence or presence of ATP. After incubation for 2 hours at 37°C, the reaction was stopped by addition of SDS-loading buffer. *In vitro* ubiquitylation was detected by SDS-PAGE followed by Western blot analysis using anti-ubiquitin antibodies. **b,** HOIL-1L and HOIP stimulate linear ubiquitylation *in vivo.* Immobilised GST-tagged ABIN-1 UBAN domain was used as bait to pull down and detect linear ubiquitylation of proteins in lysates prepared from HEK293T cells transfected with full-length HOIP and HOIL-1L. HOIP and HOIL-1L stimulates linear ubiquitylation of cellular proteins. GST-ABIN1-UBAN1 D485A, incapable of binding linear ubiquitin, was used as negative control. Ponceau S staining depicts the input of GST fusion proteins. These samples were also blotted with anti-HOIL-1L and anti-HOIP antibodies. **c**, The UBAN domains of NEMO and ABIN1 binds to linear ubiquitin chains. Purified linear di-ubiquitin (2xUb) or tetra-ubiquitin (4xUb) chains were incubated with GST (control), GST-NEMO-UBAN or GST-ABIN1-UBAN for 16 hours. After extensive washing, the GST-bound proteins were analyzed by Western blot using anti-ubiquitin antibodies. **d,** The ABIN1-UBAN domain selectively binds to linear tetraubiquitin. Purified linear and Lys63-linked tetra-ubiquitin (4xUb) chains were mixed with or without total cell lysates of wt MEFs and incubated with GST (control), GST-ABIN1-UBAN or GST-ABIN1-UBAN D485A. GST-bound proteins were analyzed by Western blot using anti-Ubiquitin antibodies. Loading of GST-fusion proteins was analyzed by Ponceau S staining. **e,** A mutation in ABIN1 abolishes linear ubiquitin binding. HEK293T cells were transfected with Myc-tagged wild type or D485A mutant ABIN-1. After 36 hours of transfection, cells were harvested for

GST-pull down assay with GST alone, or GST-tagged di-ubiquitin (2xUb) or tetra-ubiquitin (4xUb) for 16 hours. GST-bound proteins were analyzed by Western blot using anti-Myc antibodies.

Figure S4. Quantitation of linear ubiquitylation by HOIP and SHARPIN in cells. a-d, Design of the AQUA experiment and MS spectra of detected AQUA peptides. **e**, measured absolute amounts of linear-, mono-, and total ubiquitin in the whole cell lysate of HEK293 cells transfected with WT SHARPIN / WT HOIP (Lys0, Arg0) and SHARPIN TF_LV/HOIP RING (Lys4, Arg6) mutant. The results are reported as pmol/100µg of crude protein extract.

Figure S5. NEMO is a direct target of linear ubiquitylation by HOIP and SHARPIN. a, Experimental design of the SILAC experiment comparing relative expression levels of linear ubiquitin on immunoprecipitated NEMO in HEK293 cells transfected with WT HOIP/SHARPIN (Lys0,Arg0), HOIP RING mut-SHARPIN TF_LV (Lys4, Arg6) and WT HOIP/SHARPIN (Lys8,Arg10). Spectral counts (sum of all identified MS/MS events of a protein or a peptide) reflect the distribution of NEMO,ubiquitin and linear ubiquitin in the analyzed gel slices; **b**, experimental design of the SILAC MS experiment comparing relative expression levels of linear ubiquitin in HEK293 cells transfected with WT HOIP/SHARPIN (Lys0,Arg0), HOIP RING mut-SHARPIN TF_LV (Lys4,Arg6) and mock transfection (Lys8,Arg10). **c,** table showing 11 localized and quantified Gly-Gly modification sites on NEMO peptides and four Gly-Gly modification sites on ubiquitin peptides. Two additional Gly-Gly-modified lysines, K207 and K351, were detected on NEMO in qualitative MS studies.

Figure S6. Interaction between SHARPIN and NEMO is induced upon TNFα stimulation. Wild type or *Sharpin*^{cpdm}*/Sharpin*^{cpdm} MEFs were treated by TNFα for indicated

time. Total cell lysates were subjected to immunoprecipitation using anti-SHARPIN antibody. Total cell lysates and immunoprecipitates were analyzed by Western blot using anti-NEMO, anti-HOIP and anti-SHARPIN antibodies. * indicates a non-specific band recognized by anti-HOIP antibodies.

Figure S7. TNFα-induced nuclear translocation of p65 is impaired in SHARPIN and HOIL-1L deficient MEFs.

a TNFα-induced nuclear translocation of p65 is inhibited by SHARPIN and HOIL-1L deficiency. Wild type or *Sharpin*^{cpdm}/*Sharpin*^{cpdm} MEFs stably transduced with non-targeting (control) or HOIL-1L shRNA were grown on glass coverslips and starved for 16-20h before treatment with 20 ng/ml TNFα for the indicated times. The cells were fixed and stained with anti-p65 and DAPI (nucleus). Bar, 20 μm. **b,** Introduction of super-repressor IκBα mutant inhibits nuclear translocation of p65 both in wild type and SHARPIN deficient cells. Wild type or *Sharpin*^{cpdm}/*Sharpin*^{cpdm} MEFs stably transduced with super-repressor IκBα-(S32A/S36A mutant) were starved for 16-20 hours and stimulated with TNF-α (20 ng/ml) for the indicated times. Nuclear and cytoplasmic fractions were prepared and p65 nuclear translocation was analyzed by Western blot. NUP62 and GAPDH were used for fractionation markers of nuclear and cytoplasm, respectively.

Figure S8. Nuclear translocation of p65 and JNK activation is impaired in various SHARPIN deficient cells.

a, LPS-induced nuclear translocation of p65 is impaired in SHARPIN deficient macrophages. Thioglycollate-elicited peritoneal macrophages were seeded on glass cover-slips and treated with 10 ng/ml of LPS for 15 minutes. Cells were fixed and stained with anti-p65 antibody and DAPI (nucleus). Scale bar, 10 μm. **b,** IL-1β-induced NF-κB activation is partially impaired in

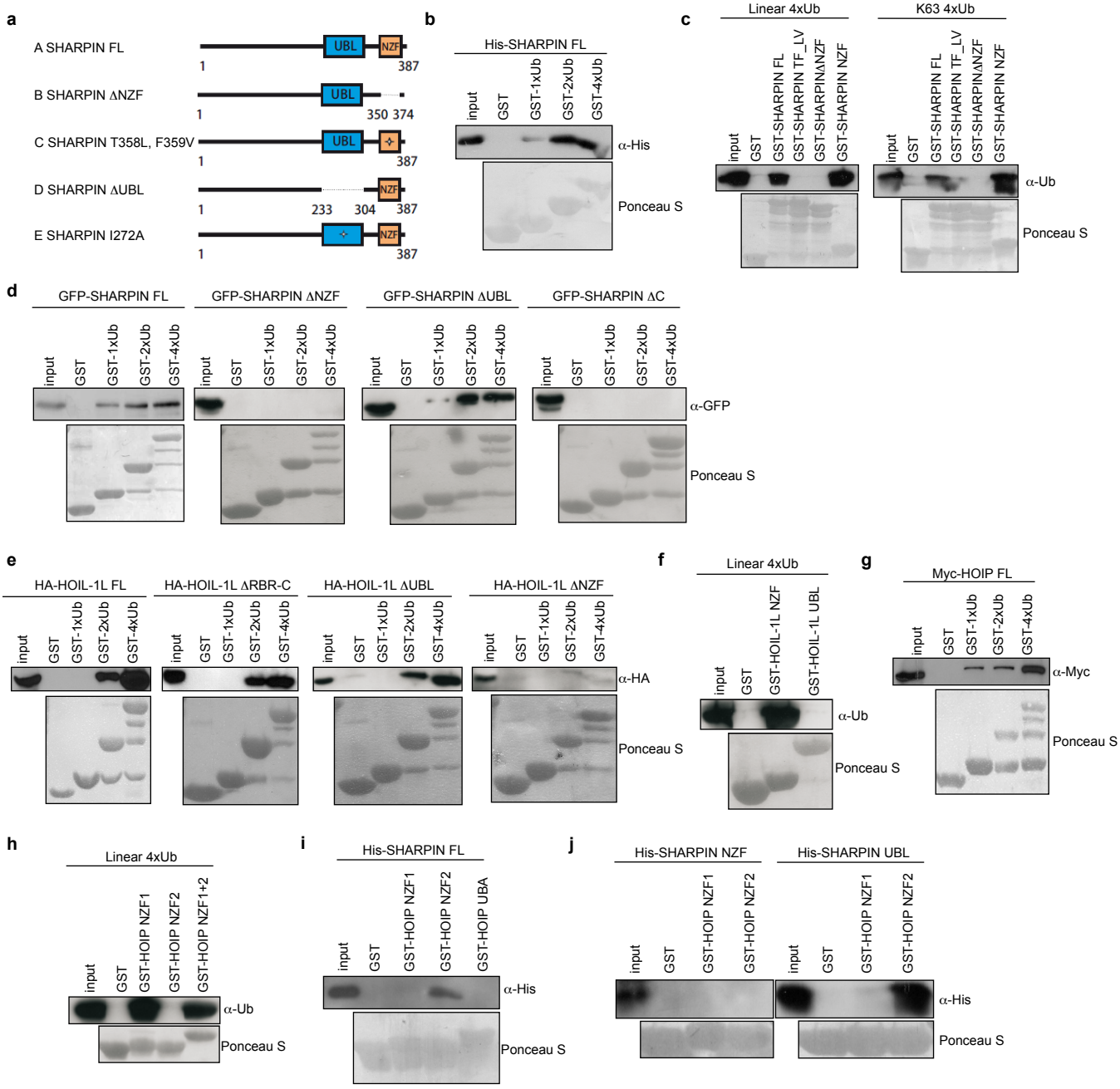
SHARPIN deficient cells. SHARPIN deficient MEFs were treated by murine IL-1β in different concentration as indicated. IL-1β-treated MEFs were harvested and total cell lysates were examined by Western blots using antibodies against IκBα, pIκBα, SHARPIN and Vinculin. Degradation and phosphorylation of IκBα were both delayed in SHARPIN deficient MEFs in all the different concentration of IL-1β treatment. **c,** Loss of SHARPIN results in impaired JNK activation in MEFs. *Sharpin*$^{+}$*/Sharpin*^{cpdm} or *Sharpin*^{cpdm}*/Sharpin*^{cpdm} MEFs were stimulated with 20 ng/ml TNFα. Cell lysates were probed with the indicated antibodies to detect activation of JNK and confirm equal protein loading. **d,** Primary splenic B-cells from *Sharpin*^{cpdm}*/Sharpin*^{cpdm} show reduced activation of JNK in response to soluble CD40 ligand stimulation. Cell lysates were probed for phosphorylated JNK to monitor activation of JNK and vinculin as loading control. **e**, Peritoneal macrophages from *Sharpin*^{cpdm}*/Sharpin*^{cpdm} mice show reduced activation of JNK compared to wild type macrophages. Cells were stimulated with LPS (10 ng/ml) for the indicated times. Lysates were probed with phosphorylated JNK antibody for detection of activation and vinculin antibody for checking equal protein loading.

Figure S9. TNFα-induced apoptosis is accelerated in SHARPIN deficient MEFs but not in HOIL-1L deficient MEFs.

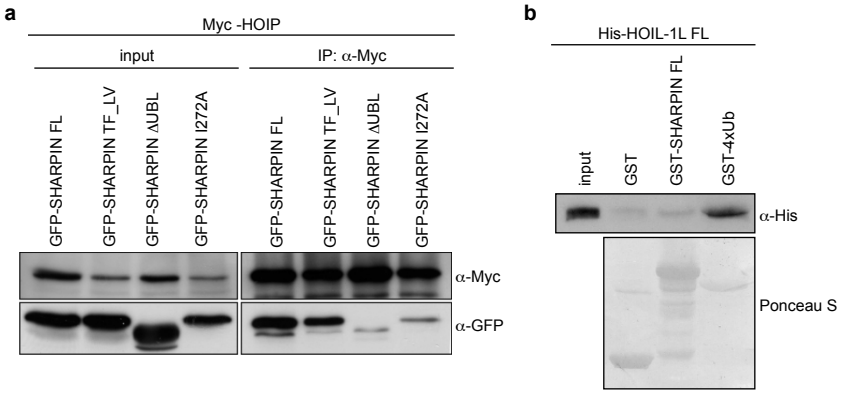
a, TNFα induces rapid morphological changes in *Sharpin*^{cpdm}*/Sharpin*^{cpdm} MEFs. Morphology of cultured *Sharpin*$^{+}$*/Sharpin*^{cpdm} and *Sharpin*^{cpdm}*/Sharpin*^{cpdm} MEFs after treatment with TNF α (10 ng/ml) and CHX (1 μg/ml) for 4 hours is shown. Arrowheads indicate cells undergoing apoptosis. **b**, A pan-Caspase inhibitor rescues TNFα-induced cell death in *Sharpin*^{cpdm}*/Sharpin*^{cpdm} MEFs. MEFs isolated from *Sharpin*^{cpdm}*/Sharpin*^{cpdm} mice were grown in 96-well plates and pretreated with zVAD-FMK (50 μM) for 30 minutes, prior to stimulation with vehicle control or TNFα (10 ng/ml) and cycloheximide (CHX) (1 μg/ml). Cell detachment and death was measured continuously in real-time using an impedance based

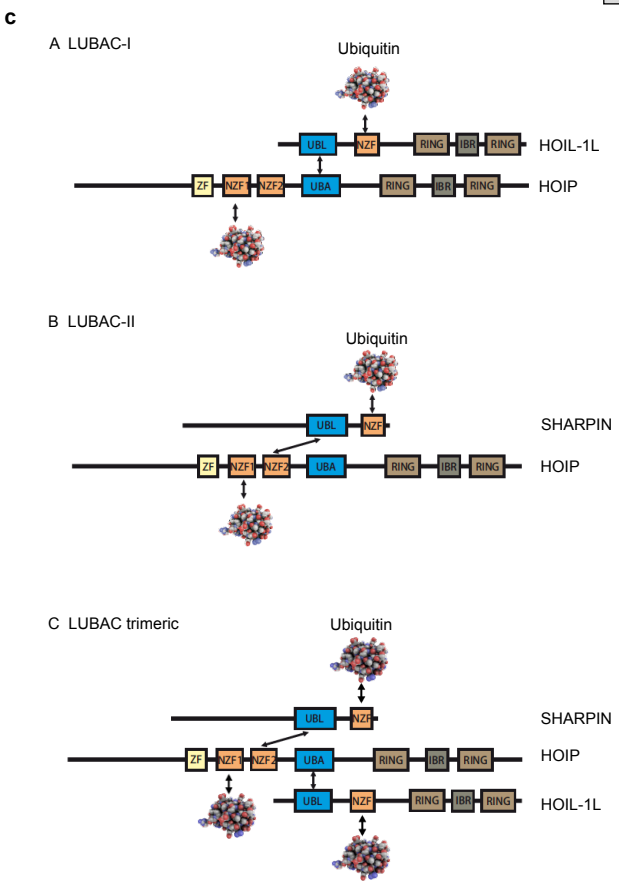
real-time cell analyzer (RTCA). The amount of surviving cells is plotted in log scale, indicating normalized cell index over time. **c**, TNFα induces activation of Caspases in *Sharpin*^{cpdm}*/Sharpin*^{cpdm} MEFs but not in *HOIL-1* -/- MEFs. Immortalized MEFs from wild type, *HOIL-1* -/- or *Sharpin*^{cpdm}*/Sharpin*^{cpdm} mice were treated with CHX (1 μg/ml) and/or TNFα (10 ng/ml). After the indicated times, total cell lysates were analyzed by Western blot using antibodies recognizing cleaved Caspase-3, HOIL-1L and SHARPIN. Anti-Vinculin antibodies were employed as loading control. **d**, HOIL-1L knockdown has no effect on TNFα-induced activation of Caspases in *Sharpin*^{cpdm}*/Sharpin*^{cpdm} MEFs. Wild type or *Sharpin*^{cpdm}*/Sharpin*^{cpdm} MEFs stably transduced with non-targeting (control) or HOIL-1L shRNA were treated with CHX (1 μg/ml) and TNFα (10 ng/ml) for the indicated times before lysis and Western blot analysis. **e, f, g**, Apoptosis in *Sharpin*^{cpdm}*/Sharpin*^{cpdm} MEFs is not enhanced by Staurosporin (e), Brefeldin A (f) or Doxrubicin (g). Wild type or *Sharpin*^{cpdm}*/Sharpin*^{cpdm} MEFs were treated by Staurosporin (0.1 μM) (e), Brefeldin A (5 μg/ml) (f) or Doxrubicin (0.5 uM) (g) for indicated time. Total cell lysates were subjected to Western blot using anti-PARP, anti-SHARPIN and anti-Vinculin antibodies.

Supplementary Figure 1

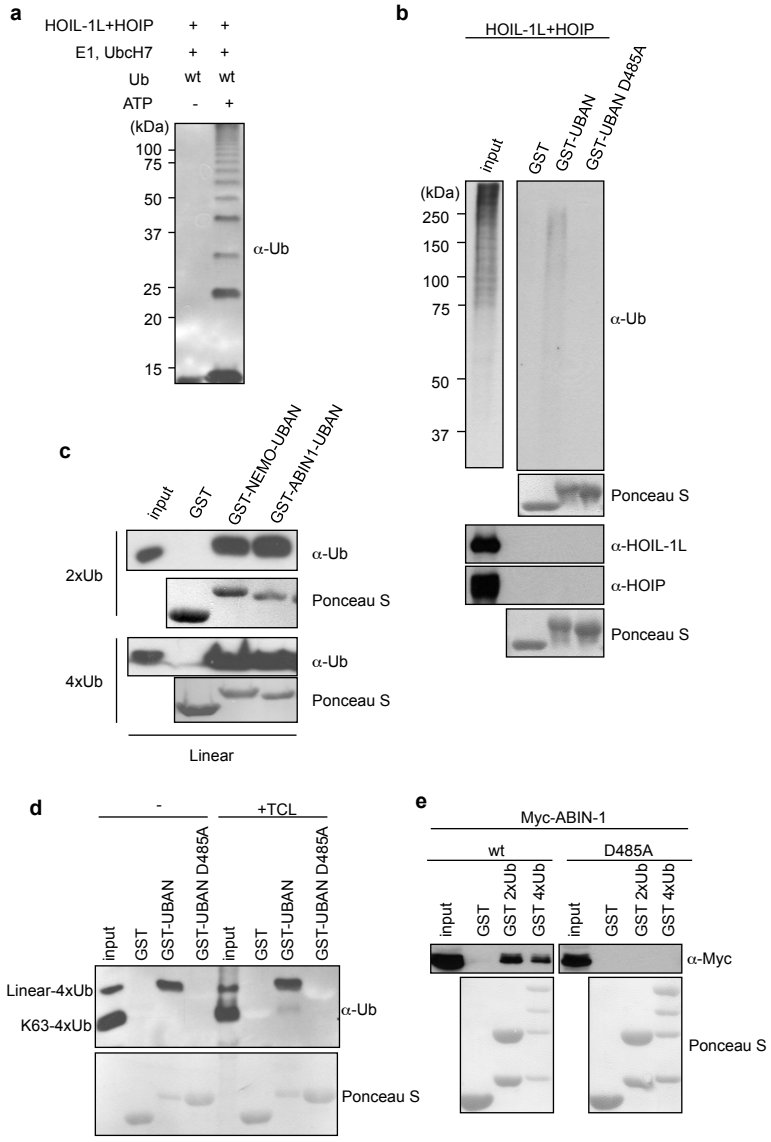


Supplementary Figure 2

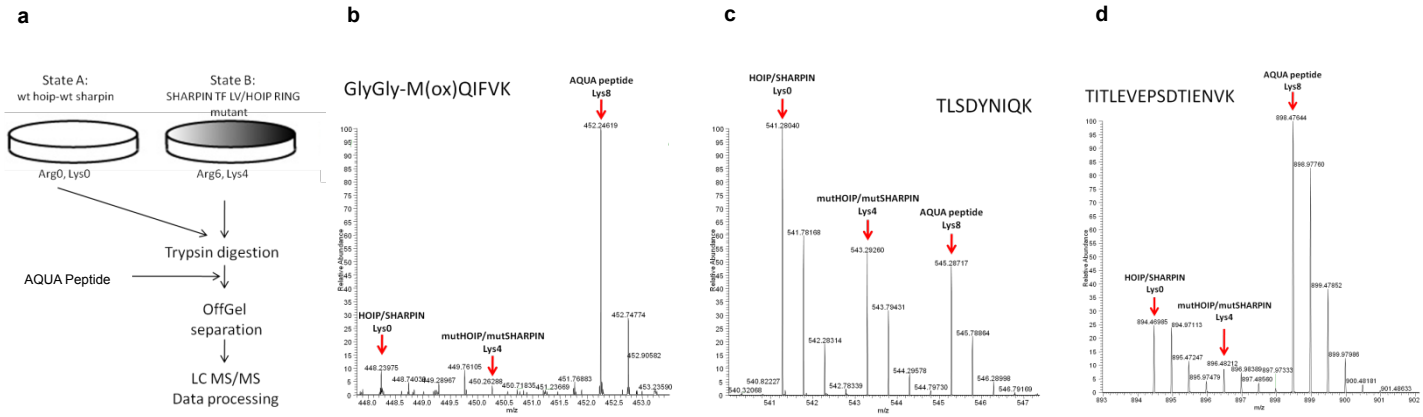




Supplementary Figure 3



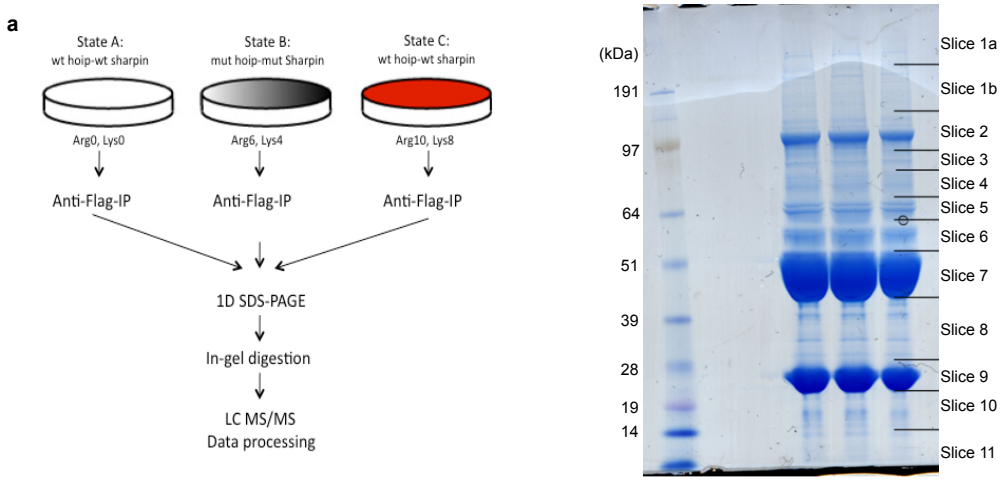
Supplementary Figure 4

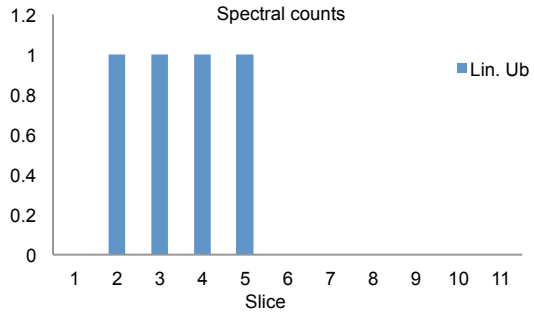


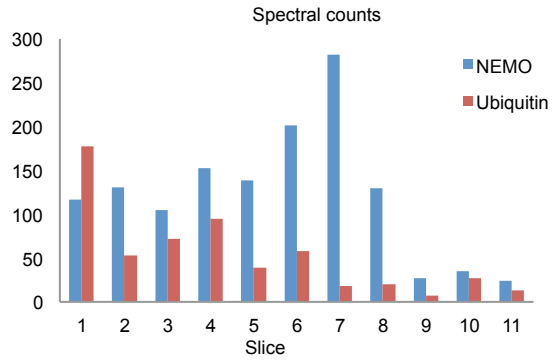
e

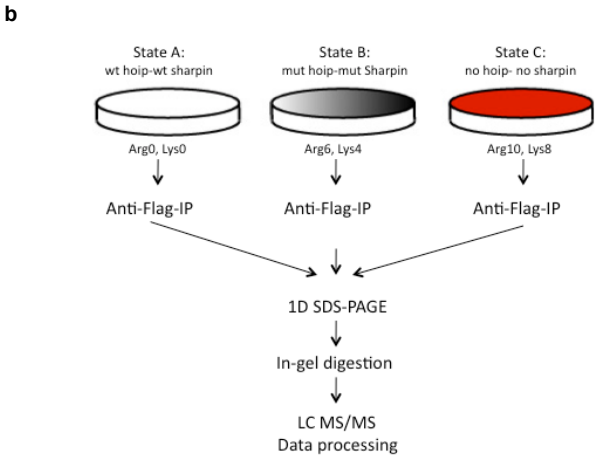
AQUA Peptide Sequence	Measured amount WT SHARPIN/HOIP (pmol)	Measured amount inactive SHARPIN/HOIP (pmol)
TITLEVEPSDTIENVK	10.72	5.75
TLSDYNIQK	12.21	8.32
GGMQIFVK	0.34	0.07

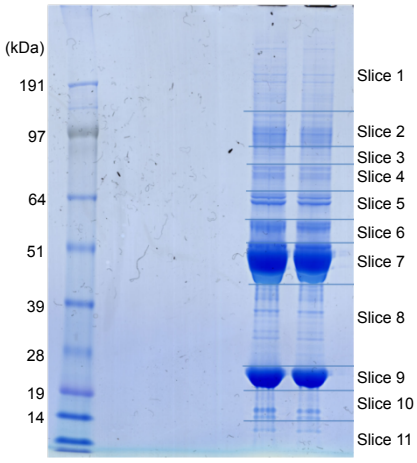
Supplementary Figure 5

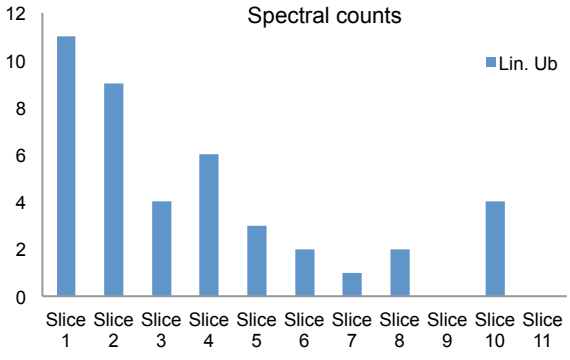


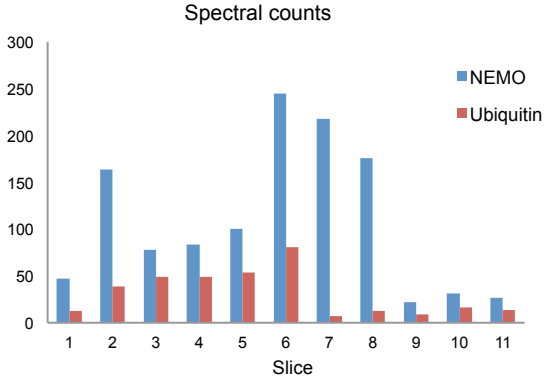












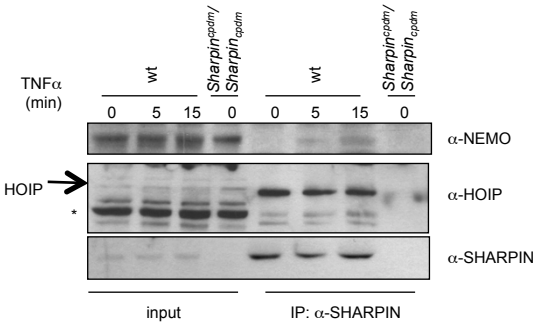
Supplementary Figure 5

c

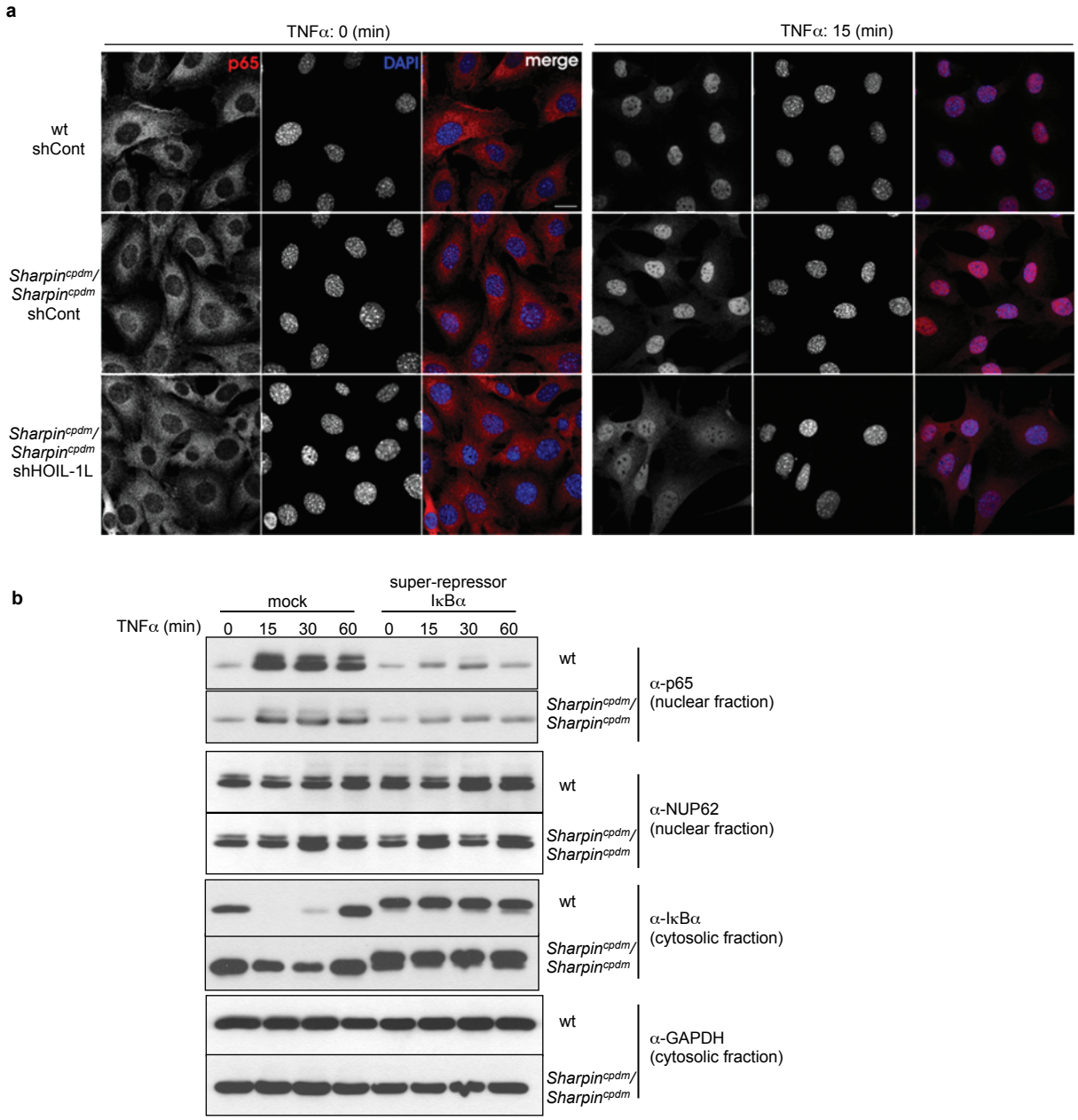
Modified NEMO sequence	GlyGly-position	SHARPIN/ HOIP („light“)	mutSHARPIN/ mutHOIP („medium“)	SHARPIN/ HOIP („heavy“)
AQADIYK(GG)ADFQAER	K377	1	0.79407	1.1359
ASVK(GG)AQVTSLLGELQESQSR	K211	1	0.43638	0.1125
K(GG)ELLQEQLEQLQR	K394	1	0.61786	0.74046
GM(ox)QLEDLK(GG)QQLQQAEEALVAK	K332	1	0.4362	1.0266
QQLQQAEEALVAK(GG)QEVIDK	K345	1	0.51333	0.91316
IVMETVPVLK(GG)AQADIYK	K370	1	0.8259	1.0361
K(GG)LAQLQVAYHQLFQEYDNHIK	K294	1	0.32363	0.73138
LAQLQVAYHQLFQEYDNHIK(GG)SSVVGSER	K314	1	0.59177	0.94499
LGLEK(GG)LDLK	K179	1	0.57256	0.67759
LK(GG)EEAEQHK	K353	1	0.53569	1.0114
QEVIDKLKEEAEQHK(GG)	K360	1	0.36654	0.81744

Modified Ubiquitin sequence	GlyGly-position	SHARPIN/ HOIP („light“)	mutSHARPIN/ mutHOIP („medium“)	SHARPIN/ HOIP („heavy“)
GG-MQIFVK	1	1	0.013293	1.4992
GG-M(ox)QIFVK	1	1	0.048098	1.5203
LIFAGK(GG)QLEDGR	48	1	0.39378	0.96244
TLSDYNIQK(GG)ESTLHLVLR	63	1	0.35182	1.3081
TLTGK(GG)TITLEVEPSDTIENVK	11	1	0.28479	0.87937

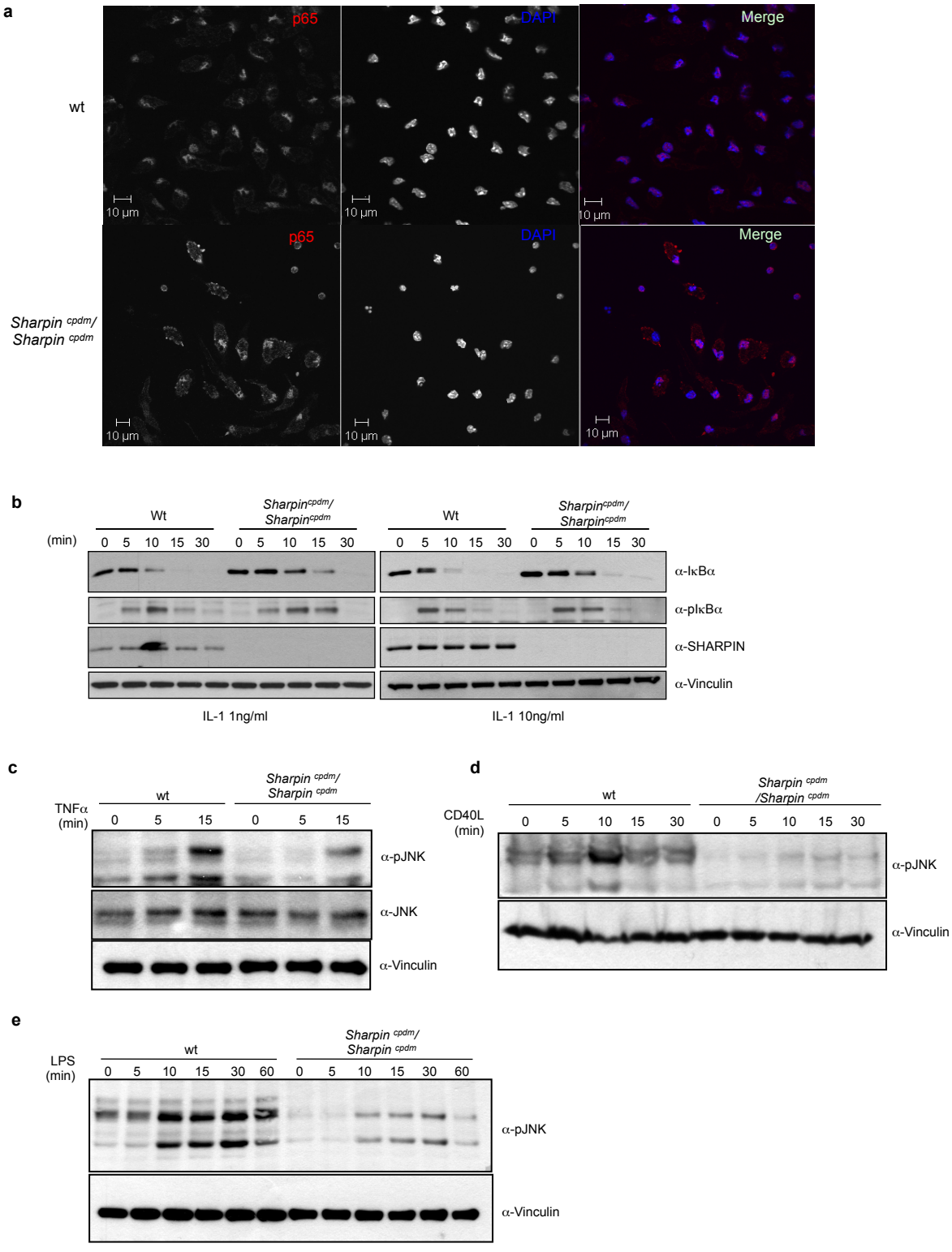
Supplementary Figure 6



Supplementary Figure 7



Supplementary Figure 8



Supplementary Figure 9